TopBP1 Regulates Human Papillomavirus Type 16 E2 Interaction with Chromatin[▽]

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Human papillomavirus type 16 (HPV16) E2 regulates transcription from and replication of the viral genome, in association with viral and cellular factors. HPV16 E2 interacts functionally with TopBP1, a cellular protein essential for the initiation of cellular, and potentially viral, DNA replication. This report demonstrates that the absence of TopBP1 results in the redistribution of HPV16 E2 into an alternative cellular protein complex, resulting in enhanced affinity for chromatin. This redistribution does not significantly alter the ability of HPV16 E2 to either activate or repress transcription. We also show colocalization of both proteins on chromatin at late stages of mitosis, suggesting that TopBP1 could be the mitotic chromatin receptor for HPV16 E2. The possible significance of the results for the regulation of the viral life cycle is discussed.

All human papillomaviruses (HPVs) encode an E2 protein which regulates the replication of and transcription from the viral genome (5). We have previously identified TopBP1 as a functional cellular interacting partner for HPV type 16 (HPV16) E2 (hereafter called E2) which increases its transcriptional and DNA replication activity (2, 3). TopBP1 is a BRCT repeat-containing protein that can regulate many aspects of nucleic acid metabolism, including transcription, replication, and DNA damage and repair processes (6, 10, 12, 14, 19–22). As both E2 and TopBP1 are chromatin-associated proteins, we investigated whether TopBP1 may be a chromatin receptor for E2 and a regulator of E2 function.

TopBP1 is not essential for E2 transcriptional activity. 293T cells (chosen due to their high transfectability and ease of TopBP1 knockdown as demonstrated below) were cultured and used in transcription assays as previously described (18). TopBP1 was depleted or mock depleted using pSUPER-TopBP1 or pSUPER plasmids (8). From the results shown in Fig. 1A, it is clear that removal of TopBP1 has little effect on the ability of E2 to activate transcription from the thymidine kinase promoter or to repress transcription from the HPV18 promoter. Thirty micrograms of protein from each lysate from the assays whose results are shown in Fig. 1A was Western blotted and probed, as described previously (3), for TopBP1 and E2 (Fig. 1B). Good depletion of TopBP1 can be observed in samples transfected with the pSUPER-TopBP1 plasmid as opposed to the control plasmid. In samples where TopBP1 is depleted, the levels of E2 are markedly elevated. Using the same protein preparation technique, we demonstrated that this increased level of E2 was not due to enhanced stability of the E2 protein (not shown). We therefore investigated the ability

of TopBP1 to alter the subcellular localization, and therefore potentially the solubilization, of E2.

Depletion of TopBP1 alters the subcellular localization of E2. 293T cells transiently transfected with E2 and TopBP1 depletion plasmids were subjected to cytoplasmic/nuclear fractionation as depicted in Fig. 1C (17). Figure 1D shows that depletion of TopBP1 results in E2 proportionally redistributing into the chromatin pellet. Sequential extraction of the chromatin pellet with increasing salt concentrations demonstrated that removal of TopBP1 results in an enhanced affinity for chromatin by E2 (Fig. 2A and B) (11). With the use of this technique for chromatin preparation, very little soluble E2 protein is detected in the absence of TopBP1 (Fig. 2B). This redistribution of E2 is not due to a cell cycle arrest in S phase as a consequence of TopBP1 depletion, as arrest of cells in S phase using hydroxyurea does not redistribute E2 (Fig. 2C). Bovine papillomavirus type 1 (BPV1) E2, which also interacts with TopBP1 (not shown), did not have its chromatin affinity significantly altered by TopBP1 depletion, as demonstrated in Fig. 2D. Recent results have demonstrated that both BPV1 E2 and HPV16 E2 use alternative mechanisms for attachment to chromatin during mitosis (13). Our results demonstrate that these two proteins also have different interactions with chromatin in interphase cells.

The results shown in Fig. 1 and 2 are apparently contradictory; in Fig. 1 there seems to be more soluble E2 in the absence of TopBP1, while in Fig. 2 there is clearly less. However, this can easily be explained if the protocol for E2 solubilization in Fig. 1 removes chromatin-attached E2. If this is the case, the fact that there was more E2 protein suggests to us that the chromatin-associated E2 is more stable than soluble E2 and that this shift to the chromatin fraction may result in more E2 protein being detected in the results shown in Fig. 1. The stability of chromatin and soluble E2 was therefore investigated as depicted in Fig. 3A, and the results are shown in Fig. 3B. The upper gel demonstrates that, in the presence of TopBP1, soluble E2 has a relatively short half-life, as we have previously demonstrated (16). However, all chromatin-attached E2 has an extended half-life. In the absence of TopBP1,

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FIG. 1. TopBP1 depletion modifies E2 subcellular localization. (A) One microgram of the indicated reporter plasmid was cotransfected with increasing amounts of HPV16 E2 and 1 μ g of depletion or mock-depletion plasmid. Cells were harvested 60 h posttransfection and assayed for luciferase activity (Promega). Luciferase activity for lysates containing no E2 was normalized to 1 and other activities were calculated as differences (*n*-fold). (B) Thirty-microgram portions of lysates from cells treated as described for panel A were Western blotted and probed for TopBP1 (upper gel) and HPV16 E2 (lower gel). (C) Outline of fractionation used for results shown in panel D. Cells were cotransfected with 1 μ g HPV16 E2 and 1 μ g depletion or mock-depletion plasmid and harvested 60 h later. The nuclei were prepared and pelleted out from the cytoplasm. The nuclei were then lysed and the nucleosol was separated from the insoluble pellet. Samples were Western blotted and probed for TopBP1, HPV16 E2, and ORC2 (predominantly nuclear marker). C, cytoplasmic; N, nuclear; P, pellet.

a different picture emerges; there is no detectable soluble E2 in the results shown in the upper gel, but the 0.2 M and 0.4 M NaCl fractions clearly show half-lives similar to that of the soluble E2 fraction in the presence of TopBP1. However, in the chromatin pellet, the stability of E2 is increased. These results demonstrate that there are two cellular pools of E2, one stable and one not. In the absence of TopBP1, the unstable fraction is shifted to be more associated with chromatin, suggesting that in the absence of TopBP1, E2 associates with alternative protein complexes. We investigated this using the protocol described in the legend for Fig. 3C that fractionates cellular complexes based on their sedimentation coefficient. In the results shown in Fig. 3D, it is clear that E2 and TopBP1 are present in the same cellular frac-

tions, suggesting that they exist in the same complex. However, when TopBP1 is significantly depleted, the E2 protein clearly shifts into fractions with higher sedimentation coefficients.

To confirm that the results observed are not due to the specific small interfering RNA (siRNA) sequence targeting TopBP1, or due to the 293T cell line, we duplicated the experiments whose results are shown in Fig. 1 using siRNA oligonucleotides targeting an alternative TopBP1 sequence in both 293T and C33a cells. The results of these experiments are shown in Fig. 4, where it is clear that two methods for knocking down TopBP1 in two different cell lines have the same results. This confirms that TopBP1 plays a specific role in regulating the cellular distribution of E2.

FIG. 2. TopBP1 depletion causes association of E2 with less-soluble biochemical fractions. (A) Outline of salt extraction method. One microgram E2 (HPV16 E2 or BPV1 E2, as indicated for panels B and D) was cotransfected with 1 μ g of either TopBP1 depletion or mock-depletion vector, and cells were harvested 60 h later. Cells were lysed in CSK buffer containing 0.5% Triton X-100, and the insoluble material was pelleted. This pellet was then serially extracted with increasing concentrations of NaCl in CSK (11), and all fractions were Western blotted. (Outline adapted from Kurg et al. [11].) (B) Shown are Western blots of HPV16 E2 fractionated as shown in panel A. The upper gel shows E2 from cells untreated with depletion vectors, the middle gel shows E2 from cells transfected with mock-depletion vector, and the lower gel shows E2 from cells transfected with depletion vector. (C) Shown are Western blots of HPV16 E2 fractionated as shown in panel A. Instead of undergoing TopBP1 depletion, cells were treated with hydroxyurea (HU) for 15 h prior to harvest (lower gel) or left untreated (upper gel). (D) Shown are Western blots of BPV1 E2 fractionated as shown in panel A. Cells were treated with mock-depletion plasmid (upper gel) or depletion plasmid (lower gel). S, soluble; P, pellet.

The results demonstrate that removal of TopBP1 results in enhanced E2 association with chromatin. One explanation is that, in normal cells, TopBP1 acts as a chromatin receptor for E2, and in the absence of TopBP1, E2 associates with higher affinity with an alternative chromatin receptor. Evidence to support this hypothesis comes from the observation that in the absence of TopBP1, chromatin-attached E2 redistributes into an alternative cellular complex (Fig. 3D). Further supporting this idea are the results shown in Fig. 1B and 4, in which an alternative mechanism for solubilizing cellular proteins resulted in enhanced levels of E2 protein being detected. This could be explained if the alternative complex in which E2 finds itself in the absence of TopBP1 is highly sensitive to disruption by this solubilization technique. So, the evidence suggests that E2 and TopBP1 exist in the same cellular complex and that TopBP1 is a chromatin receptor for E2. What is the purpose of this interaction? TopBP1 clearly does not regulate the ability of E2 to either activate or repress transcription, as is shown by the results in Fig. 1. The biological properties of TopBP1 make it more likely that it is involved in the initiation of viral DNA replication. However, knocking down TopBP1 inhibits cellular replication and entry into S phase, so it is not possible to carry out meaningful HPV DNA replication assays using TopBP1

knockdown cells. Another possible role for TopBP1 in regulating E2 function is as a chromatin receptor during mitosis. Although Brd4 is the chromatin receptor for some E2 proteins (1, 7, 23), it does not seem to be for HPV16 E2. We propose that TopBP1 is an excellent candidate to act as a mitotic chromatin receptor for HPV16 E2, as TopBP1 is associated with mitotic bodies (9, 15), and we have indeed observed colocalization of TopBP1 and E2 at late stages of mitosis (Fig. 4B). The staining pattern for HPV16 E2 is similar to patterns previously shown for other alpha HPV E2 proteins (13). In conclusion, we propose that TopBP1 is a functional interacting partner for HPV16 E2 that regulates the association of this protein with chromatin and may play a key role in mediating the DNA replication and genome segregation functions of E2. With regard to the genome segregation function, it is of note that BPV1 E2 and HPV16 E2 have different mitotic chromatin receptors (13), and we demonstrate in the results shown in Fig. 2 that the HPV16 and BPV1 E2 proteins have different interactions with the TopBP1 protein. Currently, we are attempting to isolate a mutant of E2 that fails to interact with TopBP1, a process hindered by a complex interaction between the two proteins involving three domains of TopBP1 (not shown).

FIG. 3. There are two pools of E2 in the cell. (A) Outline of fractionation method used for results shown in panel B. One microgram HPV16 E2 was cotransfected with 1 mg of either mock-depletion or depletion vector. Cells were harvested at 60 h posttransfection and cycloheximide was added to cells at 1-h intervals for the 6 h preceding harvest. The cells were then fractionated and salt extracted as described in the legend for Fig. 2A. (B) Shown are Western blots of HPV16 E2 fractions over a 6-h time course after cycloheximide addition. The left-hand panels show E2 from cells transfected with mock-depletion plasmid, and the right-hand panels show E2 from cells transfected with depletion plasmid. (C) Outline of fractionation method used for results shown in panel D. One microgram HPV16E2 was cotransfected with 1 μ g of either depletion or mock-depletion plasmid, and cells were harvested 60 h later. The cells were lysed (50 mM Tris [pH 8], 400 mM NaCl, 0.5% NP-40), and a low-speed supernatant was prepared and loaded onto a sucrose gradient (5 to 20%). This was spun in an ultracentrifuge (120 $\times$ g; 15 h; 4°C) and then fractionated. The protein was precipitated using trichloroacetic acid and then Western blotted. (D) Shown are Western blots of HPV16 and TopBP1 from sucrose gradient fractions. The upper gel shows fractions from mock-depleted cells, and the lower gel shows fractions from depleted cells.

FIG. 4. (A) TopBP1 depletion has the same effects when using a different knockdown system and in a different cell line. 293T and C33a cells were cotransfected with 1 μ g HPV16 E2 and either siRNA plasmids (pSUPER, pSUPER-TopBP1) or siRNA oligonucleotides (anti-luciferase, anti-TopBP1) as indicated. The cells were harvested, and the lysates prepared and Western blotted. Blots were probed for TopBP1 to detect knockdown (upper gels), γ -tubulin as a loading control (middle gels), and HPV16 E2 to determine the effect of TopBP1 knockdown (lower gels). In both cell lines and with both siRNA systems, E2 is increased in the TopBP1-depleted lysates. (B) TopBP1 and E2 colocalise on the chromatin and centrosomes in the late telophase. U2OS cells stably transfected with E2 were fixed and stained with TVG261 mouse anti-HPV16 E2 (1:50) and with R1180 rabbit anti-TopBP1 (1:2,000) antibodies according to the protocol described in reference 4. The DNA was stained with 4',6'-diamidino-2-phenylindole.

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